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Original Research

Human placental vascular and perivascular cell heterogeneity differs between first trimester and term, and in pregnancies affected by foetal growth restriction

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ABSTRACT

Growth-restricted placentae have a reduced vascular network, impairing exchange of nutrients and oxygen. However, little is known about the differentiation events and cell types that underpin normal/abnormal placental vascular formation and function. Here, we used 23-colour flow cytometry to characterize placental vascular/perivascular populations between first trimester and term, and in foetal growth restriction (FGR). First-trimester endothelial cells had an immature phenotype ($CD144^{+/low}CD36^{-}CD146^{low}$), while term endothelial cells expressed mature endothelial markers ($CD36^{+}CD146^{+}$). At term, a distinct population of $CD31^{low}$ endothelial cells co-expressed mesenchymal markers (CD90, CD26), indicating a capacity for endothelial to mesenchymal transition (EndMT). In FGR, compared with normal pregnancies, endothelial cells constituted 3-fold fewer villous core cells ($P < 0.05$), contributing to an increased perivascular: endothelial cell ratio (2.6-fold, $P < 0.05$). This suggests that abnormal EndMT may play a role in FGR. First-trimester endothelial cells underwent EndMT in culture, losing endothelial (CD31, CD34, CD144) and gaining mesenchymal (CD90, CD26) marker expression. Together this highlights how differences in villous core cell heterogeneity and phenotype may contribute to FGR pathophysiology across gestation.

Keywords: placenta / endothelial to mesenchymal transition / foetal growth restriction / cell heterogeneity / vascular development / high-dimensional flow cytometry

Introduction

The placenta forms the interface between the mother and foetus, exchanging nutrients and oxygen to ensure healthy foetal growth. Inadequate placental exchange function is a major contributor to the pregnancy disorder foetal growth restriction (FGR). FGR occurs in 5–10% of pregnancies, and results in deceleration or stagnation of foetal growth *in utero* because the foetus is unable to get sufficient nutrients and oxygen to meet its growth potential (Nardozza *et al.*, 2017). FGR is the second leading cause of perinatal morbidity contributing to 30% of stillbirths, and FGR babies have a higher risk of long-term health complications such as developmental delay, diabetes, and cardiovascular disease (Barker *et al.*, 2002; Froen *et al.*, 2004; Rueda-Clausen *et al.*, 2011; Nardozza *et al.*, 2017; Malacova *et al.*, 2018).

Poor placental vascular development is one feature of FGR placentae that impairs exchange. Healthy placental vascular development is a dynamic process that changes across gestation. In the first trimester, the core of placental villi contains a loose mesenchymal stroma from which endothelial cell cords emerge *de novo* (vasculogenesis), and these cords elongate and branch to form the first placental capillaries (Demir *et al.*, 2006). Pericytes are then recruited to stabilize the primitive vascular network. As gestation progresses, the villous stroma becomes more densely packed and the placental vascular network is expanded, first by branching and then by non-branching angiogenesis (Geva *et al.*, 2002). Perivascular cells, including highly contractile vascular smooth muscle cells, are involved in supporting the vascular network but also influence vascular compliance and angiogenesis later in gestation. FGR placentae are characterized by poor angiogenesis, resulting in decreased vascular density and branching, which in turn increases placental vascular resistance (Mitra *et al.*, 2000; Junaid *et al.*, 2014; Lu *et al.*, 2017; Tun *et al.*, 2019). The pathophysiology of FGR begins in the first trimester when the placental vascular network is established, but the impairment continues in later gestation as demonstrated by dysfunctional terminal vessel formation and increased arterial vessel wall thickness to lumen ratios in FGR placentae (Krebs *et al.*, 1996; Battistelli *et al.*, 2004; Junaid *et al.*, 2014).

Phenotypic cell heterogeneity reflects the range of functional capacity in an organ, delineating populations with different progenitor, angiogenic, and/or proliferative capacity and has been used to identify cells involved in functionally divergent processes such as angiogenesis or vascular regression (Dawson *et al.*, 2021). Therefore, characterizing how vascular phenotypic heterogeneity changes over gestation and in FGR may provide key insight into how normal and abnormal vascular maturation progresses in the placenta. High levels of expression of CD15 by endothelial cells, a

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marker associated with early gestation blood vessels, has been reported in FGR, suggesting that phenotypic differences in endothelial phenotype associated with an immature vascular phenotype may exist in this disorder (Challier et al., 2005; Ravikumar et al., 2019). Furthermore, in normal term placentae, flow cytometry has identified functionally divergent endothelial cell populations, including a non-proliferative $CD31^{high}$ population, a proliferative $CD31^{mid}$ population, and a $CD31^{low}$ population capable of giving rise to both endothelial and mesenchymal cells (Shafiee et al., 2018). However, whilst this demonstrates the potential importance of endothelial heterogeneity in normal/abnormal placental development, to date, vascular cell heterogeneity in FGR, or in early gestation placentae, has not been investigated.

The development and function of blood vessels is also heavily influenced by perivascular cells, a very heterogeneous group of cells that includes both pericytes and vascular smooth muscle cells. In the early stages of angiogenesis, perivascular cells can help direct and stabilize nascent vessels; whereas in later stages of angiogenesis, increased proportions of perivascular cells can reduce both angiogenic capacity and vascular compliance (Ricard et al., 2014; Eilken et al., 2017; Sweeney and Foldes, 2018). Decreased artery lumen to wall ratios, indicative of thicker vessel walls, have been reported in FGR placentae (Gnecchi et al., 2006). However, the components responsible for this structural alteration or how proportionally thicker vessel walls develop across gestation is not well understood.

Despite a recent flurry of studies employing single-cell RNA sequencing to investigate placental cell populations (Suryawanshi et al., 2018; Vento-Tormo et al., 2018), a method that has revolutionized our understanding of cell heterogeneity, RNA changes are not always representative of protein changes and studies often fail to report on the stromal/endothelial lineages of the placenta (Liu et al., 2018; Vento-Tormo et al., 2018; Pique-Regi et al., 2019). Recently, we developed a high-dimensional flow cytometry panel to explore the extent of cellular heterogeneity within the first trimester villous core, with a focus on stromal cell populations (Boss et al., 2022). Here, we capitalize on this advance in resolving the placental villous core to better understand how endothelial and perivascular heterogeneity and abundance vary both across gestation and in FGR.

Materials and methods

Experimental model and subject details

Human placentae were collected following written informed consent with approval from the Northern X Health and Disability Ethics Committee (NTX/12/06/057/AM09). Six first trimester (7.3, 8.4, 8.5, 10.1, 10.3, and 11.3 weeks of gestation) and seven term (FGR and normal) (Table 1) placentae were collected for characterization, and a further six first trimester placentae (<9 weeks of gestation) were collected for cell culture experiments. Patient details for first trimester samples (except gestation) were not collected to protect patient safety as a requirement of our ethics approval. Term placentae were collected from three FGR and four normal pregnancies following delivery (Table 1). All third trimester placentae (normal and FGR) were collected from singleton pregnancies that were uncomplicated by pre-eclampsia, hypertension, diabetes or autoimmune disease, with a maternal BMI $<30\,kg/m^2$. FGR placentae were <5th customized growth centile (which adjusts the new-born weight for maternal characteristics such as ethnicity, parity, height and weight (McCowan et al., 2007)), whilst appropriately grown gestation-matched control placentae were >25th customized growth centile.

Enzymatic digestion of villous core cells

Placental villous tissue was carefully dissected away from the foetal membranes and washed thoroughly in PBS to remove maternal blood and debris. Placental explants (~1 cm^2) were largely denuded of trophoblasts (the epithelial cells that are present around the outside of placental villi) by digestion in 10 ml/1 g tissue of Enzyme Digest Solution (1 mg/ml Dispase II (Roche, Mannheim, Germany), 0.5 mg/ml Collagenase A (Roche), and 1.5 mg/ml DNAse 1 (Sigma-Aldrich, Saint Louis, MO, USA)) in advanced-DMEM/F12 (Gibco, Grand Island, NY, USA) for 10 min at 37 °C as previously described (Boss et al., 2022). Explants were washed repeatedly in PBS and then placed in a 90 mm petri dish and finely macerated with two sterile disposable scalpels (with additional maceration applied to term explants to ensure complete digestion of this more fibrous tissue). The tissue was transferred to a 50 ml Falcon® tube with 10 ml of Enzyme Digest Solution. The Falcon® tube was briefly vortexed and then placed onto a rocker at room temperature overnight. The next morning the Falcon® tube containing the digested villous tissue and enzyme solution was diluted with 40 ml PBS and filtered through a 70 μm cell strainer (Falcon, Durham, NC, USA). The filtrate containing villous core cells was centrifuged at 220×*g* for 5 min at room temperature, and the supernatant was removed. Ten ml of 1× RBC Lysis Buffer (Biolegend, San Diego, CA, USA) was incubated with the cell suspension for 10 min to remove unwanted red blood cells. Remaining cells were centrifuged at 220×*g* for 5 min and supernatant removed prior to flow cytometry.

Staining of cells for multicolour flow cytometry

Multicolour flow cell staining was performed as previously described (Boss et al., 2022). In brief, non-specific antibody binding was blocked with 5 μl of Human TruStain FcX™ and 5 μl of True-Stain Monocyte Blocker™ (Biolegend) and then cells were stained with a master mix containing antibodies at concentrations described in Tables 2, 3, and 4. All antibodies were titrated prior to the experiment (Boss et al., 2022). Immediately prior to analysis,

Table 1. Pregnancy characteristics of foetal growth restriction and control human placental samples compared in the analysis.

	FGR (n = 3)	Normal (n = 4)	*P* value
Gestation at delivery (weeks)	38.66 ± 0.95	37.93 ± 0.68	0.521
Birthweight (g)	2523.33 ± 56.86	3101.67 ± 67	$P < 0.01$
Placental weight (g)	510 ± 52.68	570.33 ± 113.05	0.263
Customized birthweight centile (%)	2.4 ± 1.82	63.67 ± 13.78	$P < 0.001$
Crown heel length at birth (cm)	47.67 ± 0.578	49.00 ± 1.5	0.251
Head circumference (cm)	33.00 ± 0.87	34.17 ± 0.85	0.240
Maternal age (years)	30.33 ± 2.08	31.00 ± 2.63	0.966
Maternal BMI (kg/m^2)	24.67 ± 7.06	21.13 ± 3.27	0.573
Sex	2 male, 1 female	2 male, 2 female	

Mann–Whitney tests.
Data are mean ± SEM.
FGR: foetal growth restriction.

DAPI (1:5000) was spiked into FACS tubes containing stained cells.

Characterization of villous core populations by spectral flow cytometry

Panel One (Table 2) was designed to characterize placental villous core cells from both first trimester and term pregnancies. This panel included established markers for placental stromal, perivascular, endothelial, haematopoietic and cytotrophoblast cells, as well as markers that enabled the exploration of placental cell phenotypic heterogeneity. Isolated villous core cells were stained and run on a Cytek Aurora using the Cytek SpectroFlo® Software Package (Cytek, San Diego, CA, USA). Characterization of this panel has previously been described at (Boss *et al.*, 2022). Where required, fluorescence minus one experiments were employed to set gates (Supplementary Fig. S1).

FACS sorting and *in vitro* culture of first trimester endothelial cells

In order to determine whether first trimester endothelial cells were susceptible to EndMT, digested villous core cells were stained with Panel Two (Table 3) using the methods described above. Stained cells were resuspended in 500 µl of Sort Buffer (10% foetal bovine serum (FBS), 2 mM EDTA in PBS) and incubated on ice until immediately prior to sorting. $CD31^+CD34^+$ cells were sorted on an BD FACS Aria II and processed using the DIVA software package (Beckman Coulter, Brea, CA, USA). Flow cytometry gating strategies were employed to eliminate debris, doublets, dead cells, trophoblasts, and endothelial cells, before gating the populations of interest. CD31+CD34+ endothelial cells were sorted into 5 ml FACS tubes containing endothelial basal media (EGM-2 medium without supplements added, Lonza, Walkersville, MD, USA) supplemented with 10% FBS at 4°C. $CD31^+CD34^+$ cells were then seeded into 24-well plates at 3000 cells/cm^2 in EGM-2 medium and cultured in a humidified ambient oxygen incubator containing 5% CO_2 at 37°C for 7 days.

Analysis of endothelial cell differentiation

At Days 3, 5, and 7, phase-contrast images of cultured $CD31^+CD34^+$ cells were captured using a Nikon Eclipse Ti-U microscope (Tokyo, Japan). At 7 days, the *in vitro* phenotype of cells was determined using flow cytometry. To do this, cells were detached from culture flasks by incubating with TrypLE Express (Gibco), followed by blocking, and then staining with a master mix for Panel Three (Table 4) using the same general methodology described above for Panel One.

Table 2. Panel One, designed to characterize placental villous core cells from first trimester and term human pregnancies using a Cytek Aurora spectral flow cytometer.

Antibody	Fluorophore	Clone	Dose (µl)	Supplier
CD55	BB515	IA10	0.6	Beckman Coulter, Brea, CA, USA
Integrin β4	FITC	450-9D	0.6	ThermoFisher, Waltham, MA, USA
CD34	PerCP	581	0.3	BD, Franklin Lakes, NJ, USA
CD36	PerCpVio700	REA760	0.6	Miltenyi Biotec, Bergisch Gladbach, Germany
VEGFR2	PE	7D4-6	0.6	Biolegend, San Diego, CA, USA
CD271	PE/Dazzle™ 594	C40-1457	0.6	Biolegend, San Diego, CA, USA
CD142	BB700	HTF-1	1.25	BD, Franklin Lakes, NJ, USA
CD26	PE/Cy5	BA5b	0.15	BD, Franklin Lakes, NJ, USA
PDPN	PE/Cy7	NC-08	0.3	BD, Franklin Lakes, NJ, USA
CD248	Alexa Fluor® 647	B1/35	0.6	BD, Franklin Lakes, NJ, USA
CD41	APC	HIP8	0.3	Biolegend, San Diego, CA, USA
CD90	Alexa700	5E10	0.6	Biolegend, San Diego, CA, USA
CD39	BUV737	TU66	1.25	BD, Franklin Lakes, NJ, USA
ICAM1	APC/Fire750	HA58	2.5	Biolegend, San Diego, CA, USA
CD144	BV421™	55-7H1	1.25	BD, Franklin Lakes, NJ, USA
CD235a	Pacific Blue™	H1264	0.3	BD, Franklin Lakes, NJ, USA
CD31	BV480™	WM59	0.3	BD, Franklin Lakes, NJ, USA
CD45	Krome-Orange	B61840	0.3	Beckman Coulter, Brea, CA, USA
CD146	BV605™	PIH12	0.3	BD, Franklin Lakes, NJ, USA
CD117	BV650™	104D2	0.3	BD, Franklin Lakes, NJ, USA
HLADR	BV750™	L243	0.3	BD, Franklin Lakes, NJ, USA
CD73	BV785™	AD2	0.3	BD, Franklin Lakes, NJ, USA
DAPI	DAPI		1 in 5000	Akoya Biosciences, Marlborough, MA, USA

Table 3. Panel Two, designed to sort human placental endothelial cells from villous core cellson an Aria II flow cytometer.

Antibody	Fluorophore	Clone	Dose (µl)	Supplier
CD45	FITC	HI30	0.3	BD, Franklin Lakes, NJ, USA
PDPN	PE	NC-08	0.3	Biolegend, San Diego, CA, USA
CD26	PE/Cy7	BA5b	0.6	BD, Franklin Lakes, NJ, USA
CD271	PE/Dazzle™ 594	C40-1457	0.6	BD, Franklin Lakes, NJ, USA
CD144	PerCP-5.5	55-7H1	0.6	BD, Franklin Lakes, NJ, USA
CD90	Alexa700	5E10	0.6	Biolegend, San Diego, CA, USA
CD36	APC-Cy7	5-271	0.6	Biolegend, San Diego, CA, USA
Integrin β4	APC	450-9D	0.6	ThermoFisher, Waltham, MA, USA
CD31	BV480™	WM59	0.3	BD, Franklin Lakes, NJ, USA
CD73	BV785™	AD2	0.3	BD, Franklin Lakes, NJ, USA
CD34	BUV395™	581	1.25	BD, Franklin Lakes, NJ, USA
DAPI			1:200	Akoya Biosciences, Marlborough, MA, USA

Table 4. Panel Three, designed to assess differentiation of first trimester human placental cells maintained *in vitro*, using a Cytek Aurora spectral flow cytometer.

Antibody	Fluorophore	Clone	Dose (μl)	Supplier
CD34	PerCP	581	0.3	BD, Franklin Lakes, NJ, USA
CD36	PerCpVio700	REA760	0.6	Miltenyi Biotec, Bergisch Gladbach, Germany
VEGFR2	PE	7D4-6	0.6	Biolegend, San Diego, CA, USA
CD271	PE/Dazzle™ 594	C40-1457	0.6	Biolegend, San Diego, CA, USA
CD142	BB700	HTF-1	1.25	BD, Franklin Lakes, NJ, USA
CD26	PE/Cy5	BA5b	0.15	BD, Franklin Lakes, NJ, USA
PDPN	PE/Cy7	NC-08	0.3	BD,Franklin Lakes, NJ, USA
CD90	Alexa700	5E10	0.6	Biolegend, San Diego, CA, USA
CD144	BV421™	55-7H1	1.25	BD, Franklin Lakes, NJ, USA
CD31	BV480™	WM59	0.3	BD, Franklin Lakes, NJ, USA
CD45	Krome-Orange	B61840	0.3	Beckman Coulter, Brea, CA, USA
CD146	BV605™	PIH12	0.3	BD, Franklin Lakes, NJ, USA
HLADR	BV750™	L243	0.3	BD, Franklin Lakes, NJ, USA
CD73	BV785™	AD2	0.3	BD, Franklin Lakes, NJ, USA
DAPI	DAPI		1 in 5000	Akoya Biosciences, Marlborough, MA, USA

Immunocytochemistry

In order to determine the capacity of first trimester CD31+CD34+ endothelial cells to upregulate contractile markers, CD31+CD34+ cells were FACS sorted using Panel Two (as above) and seeded into 96 well plates at 2000 cells/cm^2 in EGM-2. Medium was replaced every 2–3 days. At Day 7, medium was replaced with either fresh EGM-2 or switched to advanced-DMEM/F12 supplemented with 10 ng/ml transforming growth factor (TGF)-β1 (Peprotech, Cranbury, NJ, USA) (smooth muscle differentiation medium), and cells were cultured for a further 7 days. At Day 14 of culture, immunohistochemistry for smooth muscle differentiation markers was undertaken as described previously (Boss *et al.*, 2022). Cells were fixed with methanol for 10 min, washed with PBS, then incubated in 10% normal goat serum in PBS-tween for 1 h at room temperature. Cells were incubated with primary antibodies (either 5 μg/ml of anti-Calponin (Abcam, Cambridge, UK), 1 μg/ml anti-α-smooth muscle actin (1A4 αSMA (Invitrogen, Waltham, MA, USA)) or 1 μg/ml anti-myosin heavy chain 11 (MYH-11 (Abcam, UK)) or irrelevant rabbit or mouse IgG control antibodies as appropriate (Jackson ImmunoResearch, Westgrove, PA, USA)) for 1 h at room temperature. Endogenous peroxidase activity was quenched by addition of 3% H_2O_2 in methanol for 5 minutes. A Histostain Plus Bulk Kit with AEC chromogen (Invitrogen) was used as per the manufacturer's instructions to visualize antibody binding. Nuclei were counterstained with Gills II Haematoxylin (Sigma-Aldrich).

Quantification and statistical analysis

Spectrally unmixed FCS (Flow Cytometry Standard) files were exported from SpectroFlo® and either manually analysed with FCS Express v7 (Ne Novo Software, Pasadena, CA, USA) or uploaded onto Cytobank (Beckman Coulter), a platform enabling datasets to be analysed by algorithms designed to assess high-dimensional flow cytometry datasets. In Cytobank, debris, doublets, and dead cells were excluded by manual gating then equal sampling (200 000 villous cells from each placenta were selected).

In order to compare differences in villous core populations between first trimester and term, or between FGR and normal term placentae, analysed data from FCS Express was exported to enable statistical analysis. Prism Graphpad (Boston, MA, USA) was used to investigate differences in marker expression and proportional contribution of populations using non-parametric Mann-Whitney tests, owing to small sample sizes, with SEM presented. A P value <0.05 was considered significant.

Results

Abundance and phenotype of villus core populations differ between first trimester and term

In order to investigate vascular and perivascular populations of the placental core between first trimester and term, and in FGR, a previously described 23-colour flow cytometry panel (Boss *et al.*, 2022), was employed (Panel One, Table 2). This panel included markers to exclude haematopoietic cells (CD45, CD235a) and cytotrophoblasts (ITβ4), and allowed detection of endothelial and mesenchymal heterogeneity. For flow cytometry analysis, explants from first trimester (7–12 weeks of gestation), normal term or FGR (all 37–40 weeks of gestation) villous tissue were dissected and enzymatically digested to achieve a single cell suspension for antibody staining and flow cytometry.

First, we aimed to identify the main cell populations within placental villous core and to compare their relative abundance between first trimester and term placentae. To do this, we employed FlowSOM, an unbiased method used to group cells together based on high-dimensional protein expression (Van Gassen *et al.*, 2015). Cell groupings were then predicted based on known marker expression. Here, villous core cells from term placentae (n = 4) were compared with those from first trimester placentae (n = 6, expanded from our first trimester cohort previously published in Boss *et al.* (2022)). Villous core cells were grouped into six subsets as previously described (Boss *et al.*, 2022), and as follows: cytotrophoblasts by expression of ITβ4 (James *et al.*, 2015); haematopoietic cells by CD45 and CD235a (Van Handel *et al.*, 2010); endothelial cells by co-expression of CD31 and CD34 (Shafiee *et al.*, 2018); perivascular cells by expression of CD146 or CD271 (Suryawanshi *et al.*, 2018); stromal connective tissue cells by expression of either CD26 or CD90 (Suryawanshi *et al.*, 2018); and a mesenchymal population identified in first trimester placentae by co-expression of CD73 and CD90 (Fig. 1). We previously reported that Subset Six was reduced in abundance after 9 weeks of gestation (Boss *et al.*, 2022) and in line with this, here we showed that this population of mesenchymal cells was absent from the term villous core.

The proportion and heterogeneity of extravascular stromal cells changes between first trimester and term

In first trimester placentae, the majority of villous core cells were identified as extravascular stromal connective cells (Subset Five)

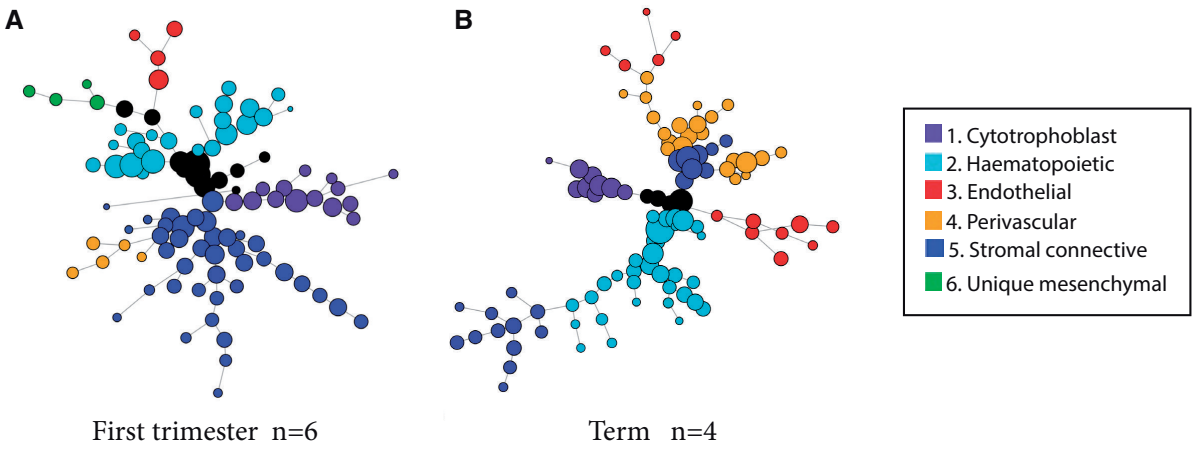

Figure 1. Minimum spanning tree plots created in Cytobank using the algorithm flowSOM. (**A**) Human first trimester villus core cells (combined plot from n = 6 samples) and (**B**) human term villus core cells (combined plot from n = 4 samples) were clustered based on marker expression similarities and then manually classified into subsets based on known marker expression. (1) cytotrophoblasts (ITβ4), (2) haematopoietic (CD45, CD235a), (3) endothelial (CD31, CD34), (4) perivascular (CD271, CD146), (5) stromal (podoplanin, CD36), and (6) unique mesenchymal (CD73, CD90). The size of each circle is directly proportional to number/proportion of cells in each cluster. FlowSOM is an unbiased method used to group cells together based on high-dimensional protein expression.

(whereas at term, these cells made up a much smaller proportion of the total core cells). Extravascular stromal cells could be further split into podoplanin$^+$CD36$^+$CD142$^+$CD26$^+$ cells (by gating podoplanin and CD36), or cells expressing only CD26$^+$ or CD90$^+$ (Fig. 2), which we have suggested are proliferative/migratory stromal or myofibroblast-like cells, respectively (Boss *et al.*, 2022). Here, we found that there was a significantly greater proportion of podoplanin$^+$CD36$^+$CD142$^+$ cells in first trimester villous core digests (47.75 ± 16.97%) than in term villous core digests (4.37 ± 3.76%) (<0.05). Conversely, the proportions of CD26$^+$/CD90$^+$cells in villous core digests were similar across gestation (31.28 ± 12.55% in the first trimester, 24.83 ± 9.66% at term). Expression of the complement control protein CD55 was observed in some podoplanin$^+$CD36$^+$ cells, but this was not consistent between samples (Fig. 2).

Vascular/perivascular heterogeneity increases between first trimester and term in line with increasing vascular specialization and maturation

As endothelial and perivascular cells are key populations involved in vascular development, characterization of these cells in the placenta between first trimester and term was a main focus of this work. Therefore, cytotrophoblasts (considered contaminants from the digestion process), and haematopoietic cells (not the focus of this work), were excluded from the analysis (Fig. 3A). Following this, CD31$^+$CD34$^+$ endothelial cells constituted 3.92 ± 2.49% of first trimester, and 10.83 ± 1.60% of term villous core cells. Phenotypic variation was observed within CD31$^+$CD34$^+$ endothelial cells, and this also changed between first trimester and term. CD144, often considered a constitutive endothelial marker, showed varied expression, with both CD144$^{low/-}$ and CD144$^{+/high}$ endothelial cell populations present in both the first trimester and at term, with a trend to increased proportions of CD144$^{low/-}$ cells in first trimester placentae (Fig. 3). Furthermore, in the first trimester, significantly fewer CD31$^+$CD34$^+$ endothelial cells expressed the anti-angiogenic marker CD36, or the vascular/perivascular adhesion receptor CD146, compared to those from term placentae ($P < 0.05$). Taken together, these results demonstrate that CD31$^+$CD34$^+$ endothelial cells from first trimester placentae broadly exhibit a phenotype more associated with immature vasculature (CD144$^{low/-}$CD36$^-$CD146^{low}) compared to those from term placentae (CD144$^+$CD36$^+$CD146$^+$) (Fig. 3D).

In the first trimester, there was a single cluster of CD31$^+$CD34$^+$ endothelial cells observed on the minimum spanning tree plots. However, in term placentae, two clusters of CD31$^+$CD34$^+$ endothelial cells were evident (Fig. 1). Of particular interest was the cluster not evident in the first trimester, which correlated with a subpopulation of CD31^{low} cells that co-expressed mesenchymal markers (CD90, CD26) (Figs 1 and 3), and constituted 33.54% of total endothelial cells. Further analysis showed that this population was present in small numbers in the first trimester (3.15%, significantly less than term $P < 0.05$), and these lower proportions meant it did not cluster as a separate population by FlowSOM in early gestation samples. As the CD31^{low} population expresses CD90 and CD26, the greater proportion of these cells in term placentae also resulted in a higher proportion of term villous core cells expressing CD90 and CD26 (Fig. 3E).

In line with more mature/established blood vessels in the placenta later in gestation, perivascular cells made up a higher proportion of the total villous core cells at term (67.24 ± 9.00%) than in the first trimester (10.30 ± 4.7%, $P < 0.01$). This increase in perivascular cell proportions resulted in an increase in the perivascular: endothelial cell ratio from ~2:1 in the first trimester to ~6:1 at term. In first trimester perivascular cells, 1.80 ± 2.24% exclusively expressed CD271 (a marker also used to identify progenitor cells), 1.83 ± 2.52% exclusively expressed CD146, and 4.35 ± 4.94% co-expressed both markers. Interestingly, in the first trimester, there was a subpopulation of perivascular cells that co-expressed CD36, CD142 and podoplanin (markers expressed by Subset Five (stromal cells)) that were not present/very rare at term (Fig. 4). The co-expression of markers expressed by stromal cells could indicate this population represents a transitional state between stromal and perivascular cells. At term, cells identified by the expression of CD146 could include vascular smooth muscle cells and pericytes associated with either large or smaller mature vessels, whereas in first trimester placentae, CD146 cells likely represent pericytes associated with developing small vessels. In line with this, CD146 expression in the first trimester demonstrated one expression level, whilst at term CD146^{high} and CD146^{low}

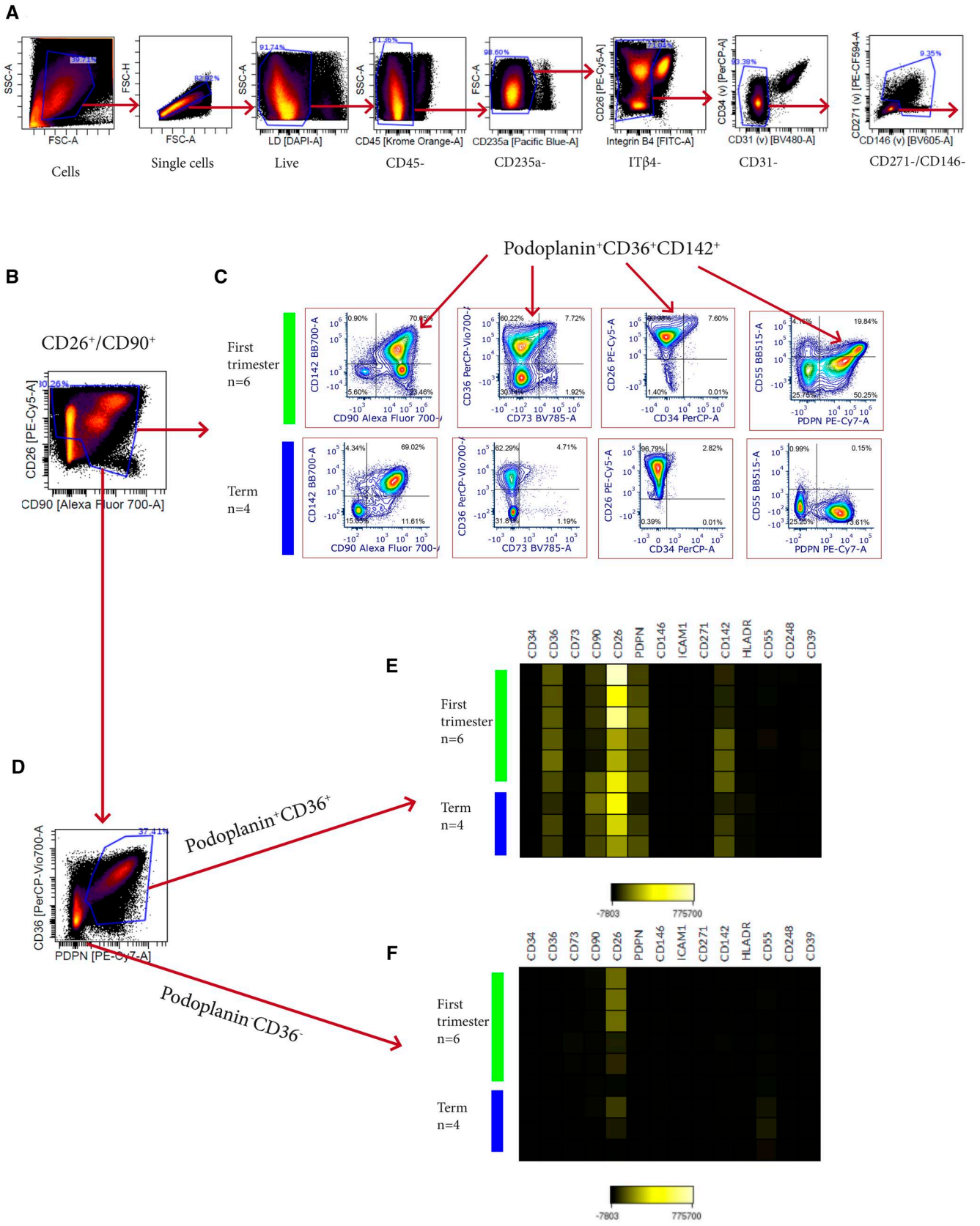

Figure 2. Extravascular stromal cell populations from first trimester and term placental villous core digests. (**A**) Flow plots demonstrating the gating strategy applied to remove debris, doublets, haematopoietic cells, cytotrophoblasts, endothelial cells, and perivascular cells. (**B**) CD26+/CD90+ gate. (**C**) Representative flow plots demonstrating expression of CD26+/CD90+ cells in first trimester and term. (**D**) Gating strategy used to identify podoplanin+CD36+ and podoplanin-CD36– cells. (**E**) A heat map comparing antigen expression between first trimester (green) and term (blue) podoplanin+CD36+ cells. (**F**) A heat map comparing antigen expression between first trimester (green) and term (blue) podoplanin-CD36– cells. SSC-A: side scatter area; FSC-A: forward scatter area; LD: live/dead.

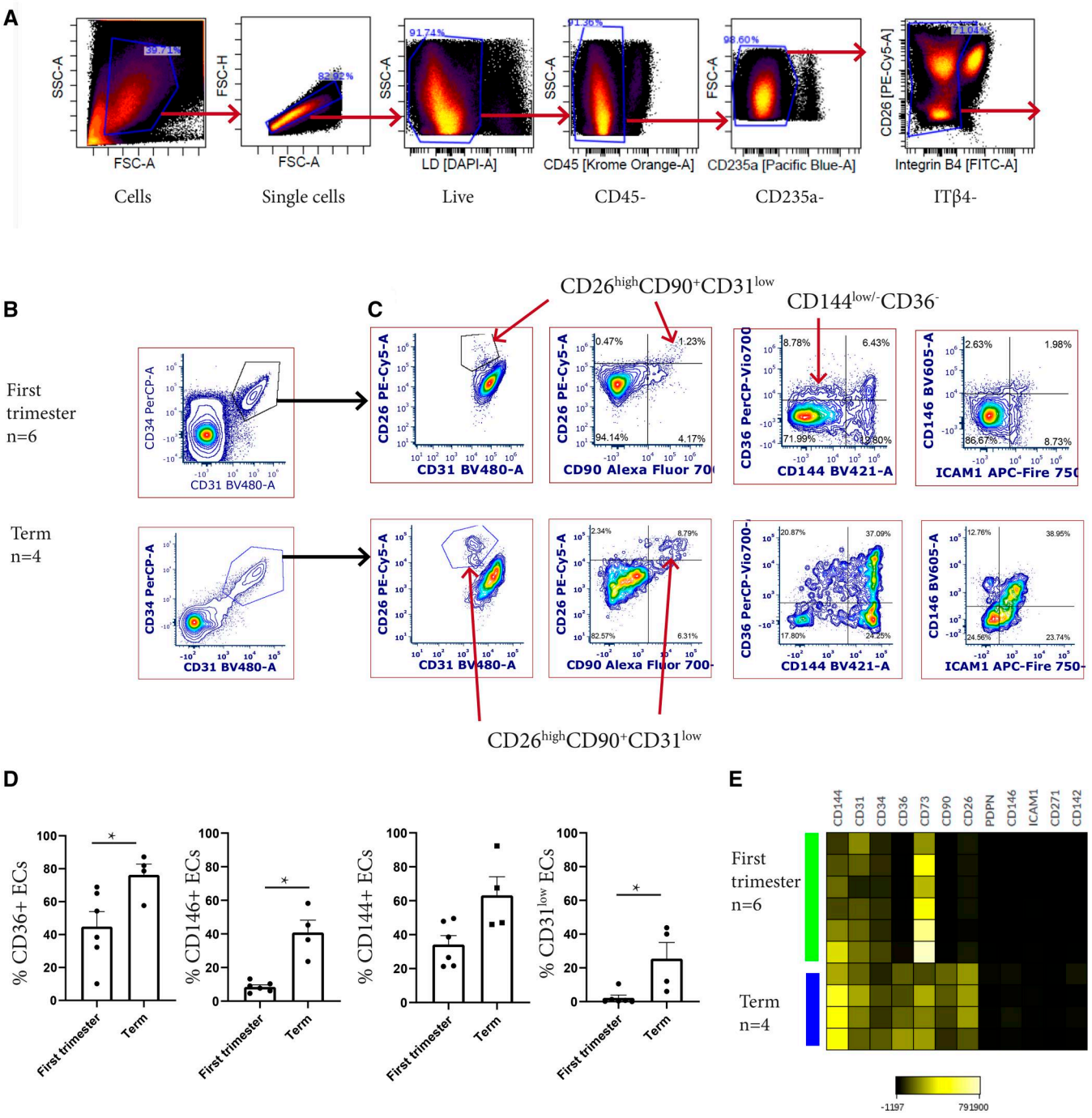

Figure 3. Endothelial cells from human first trimester and term placental villous core digests. Representative flow plots demonstrating (**A**) the gating strategy applied to remove debris, doublets, dead cells, haematopoietic cells, and cytotrophoblasts. (**B**) CD31+CD34+ endothelial cell gate. (**C**) endothelial heterogeneity at first trimester and term. (**D**) Bar graphs demonstrating percentage of endothelial cells in first trimester (green) and term (blue) villous core digests expressing CD36, CD146, CD144, and CD31low, and (**E**) a heat map comparing antigen expression between endothelial cells in first trimester (green) and term (blue) villous core digests. Mann–Whitney tests compared first trimester and term placental cells and SEM is presented. *$P < 0.05$. SSC-A: side scatter area; FSC-A: forward scatter area; LD: live/dead.

populations were present, suggesting functional differences in CD146 expression at different levels of the vascular tree (Fig. 4).

Placentae from foetal growth restricted pregnancies have a depleted proportion of endothelial cells and an increased ratio of perivascular cells to endothelial cells

Next, we examined whether differences in either phenotypic heterogeneity or proportions of villous core cells differed in placentae from FGR pregnancies compared to normal term placentae. Overall, there was a significantly lower proportion of $CD31^{+}CD34^{+}$ endothelial cells in villous core digests from FGR placentae (3.45 ± 0.49%) in comparison to normal term villous core digests (10.83 ± 1.60%, $P < 0.05$) (Fig. 5A and B). However, there was no difference in the proportion of perivascular cells (identified by $CD271^{+}/CD146^{+}$) between normal and FGR placentae (67.24 ± 9.51% and 56.93 ± 10.13%, respectively). Together, this created a significantly higher ratio of perivascular to endothelial

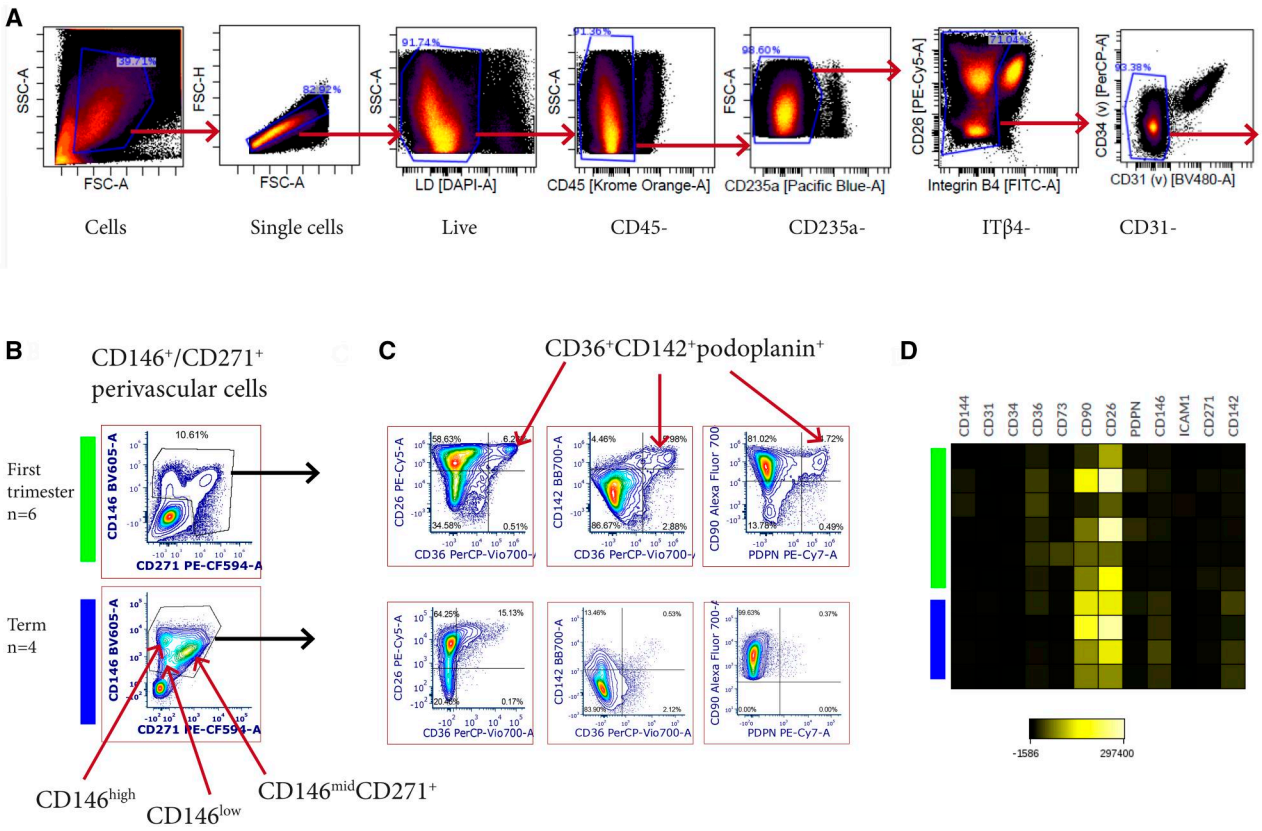

Figure 4. Perivascular cells from first trimester and term villous core digests. (**A**) Flow plots demonstrating the gating strategy applied to remove debris, doublets, haematopoietic cells, cytotrophoblasts, and endothelial cells. (**B**) Gating of CD146+/CD271+ perivascular cells. (**C**) Representative flow plots demonstrating perivascular cell heterogeneity in first trimester and term samples. (**D**) A heat map comparing antigen expression between first trimester (green) and term (blue)-derived endothelial cells. Arrows indicate the CD36+CD142+podoplanin+subpopulation that is clearly evident in the first trimester but largely absent at term. SSC-A: side scatter area; FSC-A: forward scatter area; LD: live/dead; EC: endothelial cell.

cells in FGR (~16:1) compared to normal (~6:1) placentae (Fig. 5B).

The second key difference was that while minimum spanning tree plots, created in FlowSOM, demonstrated two clusters of endothelial cells in normal term placentae (correlating with $CD31^{low}CD26^{high}CD90^{+}$ cells, and $CD31^{high}CD26^{-}CD90^{-}$ cells) (Fig. 5A), in FGR placentae, the $CD31^{low}$ endothelial cell population did not cluster separately in FlowSOM analysis. This appears to be a result of an overall reduction in the percentage of endothelial cells, meaning that all endothelial cells grouped as a single cluster in the analysis of FGR placentae (Fig. 5A). There was no difference in the proportion of $CD31^{high}CD26^{-}CD90^{-}$ endothelial cells from normal (25.25 ± 19.0%) or FGR (32.33 ± 20.5%) villous core digests. Similarly, no differences were observed in the proportions of cells expressing CD36, CD146 or CD144 between villous core digests from FGR and normal term placentae.

First trimester endothelial cells undergo EndMT and could contribute to perivascular lineages in the villous core

$CD31^{low}$ endothelial-like cells have previously been identified in term placentae, and gave rise to *in vitro* cultures of endothelial and mesenchymal cells (Shafiee *et al.*, 2018). This population was hypothesized to represent a bipotential progenitor. However, an alternative hypothesis is that this population represents endothelial cells capable of, or predisposed to, endothelial to mesenchymal transition (EndMT). EndMT is observed in normal vascular development, but in excess has been correlated with cardiovascular pathologies, by increasing vessel wall thickness and impairing angiogenic branching (Evrard *et al.*, 2016; Kovacic *et al.*, 2019). As the ability of first trimester endothelial cells to undergo EndMT has not previously been reported, here we sought to determine whether first trimester endothelial cells (key for establishing the placental vascular network) are able to undergo EndMT. To do this, placental $CD31^{+}CD34^{+}$ endothelial cells (n = 3, 7–9 weeks gestation) were FACS sorted using Panel Two (Table 3), and directly plated in an endothelial growth medium (EGM-2). Within 3 days, endothelial cells had begun to take on a spindle-shaped mesenchymal morphology and by 7 days all cells resembled spindle-shaped mesenchymal cells in the cultures (Fig. 6). When the cultured cells were assessed by flow cytometry at the end of 7 days of culture using Panel Three (Table 4), >95% of cells had lost expression of endothelial markers ($CD31^{+}CD34^{+}CD144^{+}$) and gained expression of mesenchymal associated markers ($CD26^{+}CD90^{+}$) (Fig. 6).

We observed that 8.48% of freshly isolated $CD31^{+}CD34^{+}$ first trimester endothelial cells expressed the perivascular marker CD146 (Fig. 3). However, after 7 days of culture 79.42% of sorted $CD31^{+}CD34^{+}$ endothelial cells expressed CD146, leading us to hypothesize that these mesenchymal cells may contribute to the perivascular niche in the placenta. In order to investigate whether CD146 expression was indicative of the capacity to further differentiate to a contractile-like perivascular cell, we then cultured $CD31^{+}CD34^{+}$ endothelial cells in EGM-2 for 7 days (as above, resulting in a mesenchymal phenotype) before transitioning to smooth muscle differentiation medium (advanced-DMEM/F12 + 10 ng/ml TGF-β1) for a further 7 days. Compared to cells maintained in EGM-2 medium, cells cultured in smooth muscle

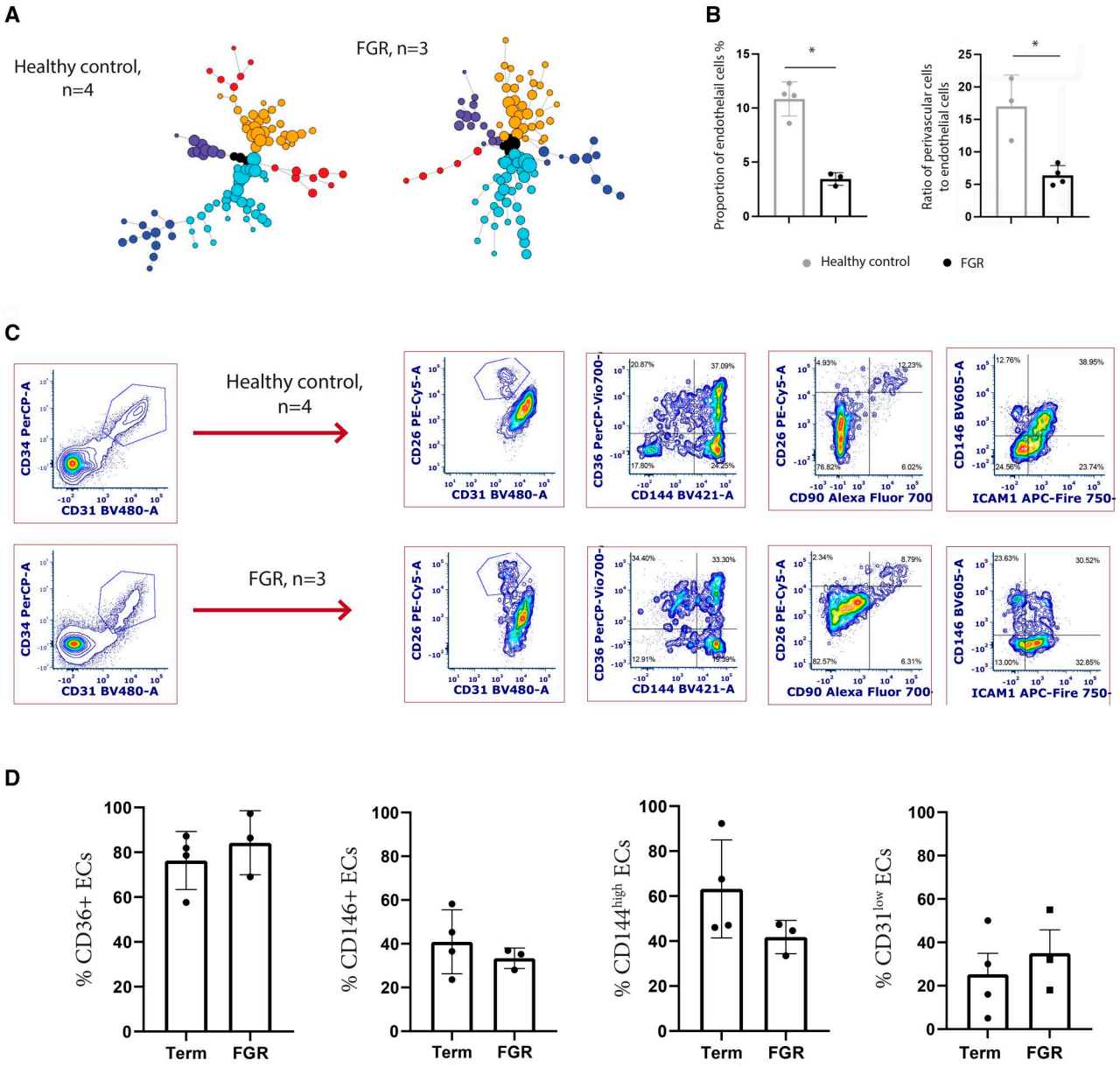

Figure 5. Vascular and perivascular cell populations from foetal growth restricted placentae. (**A**) Minimum spanning tree plots compare amalgamated villous core digests from normal term (n = 4) and FGR (n = 3) placentae. $CD34^{+}CD31^{+}$ endothelial cells have been circled. (**B**) Bar graph demonstrating the proportion of endothelial cells and the ratios of perivascular to endothelial cells in FGR and normal term digests. (**C**) Representative flow cytometry plots demonstrating the expression of key markers in $CD34^{+}CD31^{+}$ endothelial cells from normal term and FGR villous core cells. (**D**) Bar graph comparing the proportion of endothelial cells with CD36, CD146, CD144, or $CD31^{low}$ expression between normal term and FGR villous core digests. Statistical differences were assessed using Mann–Whitney tests. Error bars represent SEM. *$P < 0.05$. FGR: foetal growth restriction; EC: endothelial cell.

differentiation medium upregulated αSMA and calponin, but not the mature smooth muscle marker MYH-11 (Fig. 7).

Discussion

This study employed spectral multicolour flow cytometry to interrogate protein level heterogeneity within cell populations digested from the placental villous core between first trimester and term, and in FGR pregnancies. We demonstrated heterogeneity within placental endothelial, perivascular and stromal lineages between first trimester and term that correspond to maturation of the placental villous core and vasculature. The cellular phenotypic shifts identified in this work allow us to predict the lineage pathways involved in development, but also infer how these pathways may lead to poor vascular development in FGR. Our combined data demonstrate changes in villous core cell phenotypes and abundance between first trimester and term that align with prior reports of both the dramatic increase in vascular density, and maturation (involving gain of larger contractile vessels) of the vascular network throughout pregnancy (Mayhew, 2002; Zhang *et al.*, 2002). In FGR, we observed a lower proportion of placental endothelial cells, and a higher ratio of perivascular to endothelial cells, corresponding to the reduced vascular density and decreased vascular compliance associated with this pathology (Gong *et al.*, 2011; Junaid *et al.*, 2014, 2017). Finally, we demonstrated the capacity of first trimester endothelial cells to undergo EndMT, highlighting a potential mechanism that could contribute to altered placental vascular structure to explore in future studies.

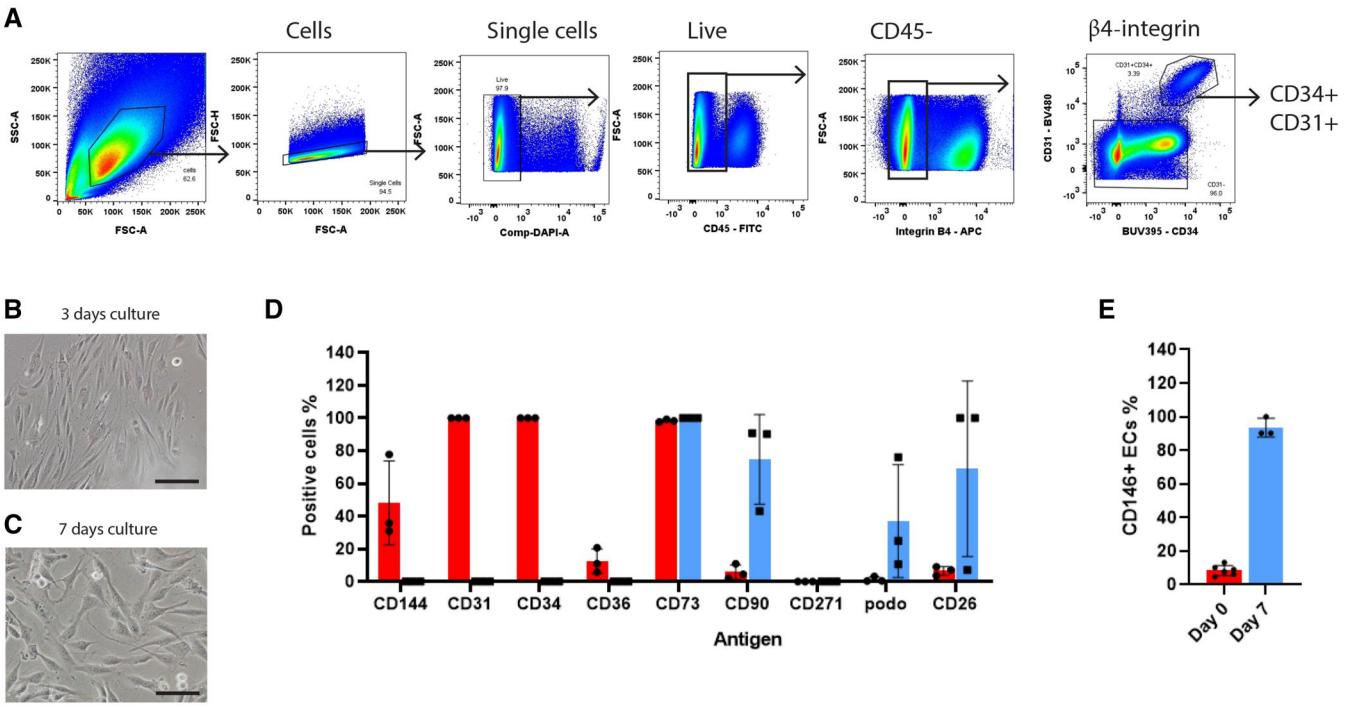

Figure 6. Endothelial to mesenchymal transition in first trimester endothelial cells. (**A**) The gating strategy applied to select CD31+CD34+ endothelial cells from first trimester villous core digests. (**B** and **C**) Photomicrographs of cultured endothelial cells at Day 3 (**B**) and Day 7 (**C**) of culture. Scale bar = 50 μm. (**D**) Bar graph depicting the expression of endothelial and perivascular markers at Day 0 (measured during FACS isolation with staining for Panel Two) and after 7 days culture (Panel Two). (**E**) Bar graph depicting CD146 expression on endothelial cells analysed from freshly isolated cells using Panel One (Day 0, n = 5) or at 7 days after culture using Panel Three (Day 7, n = 3). Bars represent SEM. SSC-A: side scatter area; FSC-A: forward scatter; EC: endothelial cell.

Focusing in on differences in the abundance and heterogeneity of extravascular stromal populations, first, we confirmed our finding that $CD73^{+}CD90^{+}$ cells were abundant prior to 9 weeks gestation (Boss *et al.*, 2022), but showed that at term, this population was all but absent, suggesting a functional role specific to early pregnancy when the nascent vascular tree is rapidly expanding. Second, $podoplanin^{+}CD36^{+}CD142^{+}$ cells were present in first trimester but significantly reduced in abundance in third trimester. This population was previously hypothesized to represent migratory, proliferative cells involved in villus branching and expansion, and potential facilitation of Hofbauer (placental macrophage) migration (Boss *et al.*, 2022), and thus their reduction in late gestation may be in line with reduced rates of villous growth in later pregnancy. Finally, the $CD90^{+}CD26^{+}$ population of extravascular stromal cells present in both first trimester and term placentae aligns with a population of cells, previously reported in single-cell RNA sequencing experiments, that co- express calponin and high levels of α-SMA, supporting the concept that these cells are myofibroblasts (Suryawanshi *et al.*, 2018).

We then turned our attention to vascular cell lineages and what their phenotypic characteristics may tell us about normal and abnormal placental vascular development. Our previous work showed that first trimester perivascular cells express CD271 and CD146 (Boss *et al.*, 2022), and this is supported by single-cell RNA sequencing data (Suryawanshi *et al.*, 2018). Here, the proportion of $CD146^{+}$ and/or $CD271^{+}$ perivascular cells was greater at term than in the first trimester (in line with increased vascular density), but these perivascular cells increased at a higher rate than endothelial cells, resulting in an increased ratio of perivascular cells to endothelial cells at term. This likely reflects a shift from immature capillaries with few pericytes in early pregnancy, to an increased proportion of stable, mature arterioles and arteries with a well-established perivascular layer at term (Mayhew, 2002; Zhang *et al.*, 2002). Interestingly, a $CD271^{+}CD146^{low/mid}$ perivascular population (indicative of more immature capillary pericytes) was present at both gestational timepoints, but a $CD271^{-}CD146^{high}$ population (previously associated with vascular smooth muscle cells in other tissues) was present only at term, further reflecting an increased degree of vascular maturity at term (Smyth *et al.*, 2018).

Increases in $CD31^{+}CD34^{+}$ endothelial cell abundance, and expression of CD36, CD144, and CD146 between first trimester and term placentae further illustrate maturation and expansion of the placental vasculature across gestation. First trimester endothelial cells lack markers associated with mature endothelial junctions (occludin, claudin-1, claudin-2, and plakoglobin), which may allow for vessel remodelling and permeability (Leach et al., 2002). We found that very few endothelial cells in the first trimester, but most endothelial cells at term, expressed CD36 (a receptor for the antiangiogenic factor thrombospondin-1 (TSP-1)) and CD146 (an adhesion molecule involved in regulating angiogenesis, and vascular integrity (Leroyer *et al.*, 2019)). Blockade/inhibition of CD36 or TSP-1 increases angiogenesis *in vitro* and *in vivo*, and low CD36 expression is observed on capillary sprout-specific endothelial cells in mice (Dawson *et al.*, 1997; Anderson *et al.*, 2008; Ren *et al.*, 2011). Thus, limited expression of CD36 on first-trimester endothelial cells could help promote vessel sprouting during branching angiogenesis. Conversely, at term, the wider degree of endothelial cell CD36 expression could indicate a mature vessel phenotype with limited capacity for branching angiogenesis.

In comparison to normal term placentae, the abundance of $CD31^{+}CD34^{+}$ endothelial cells in the villous core of FGR placentae was reduced approximately three-fold (from 10.83% to

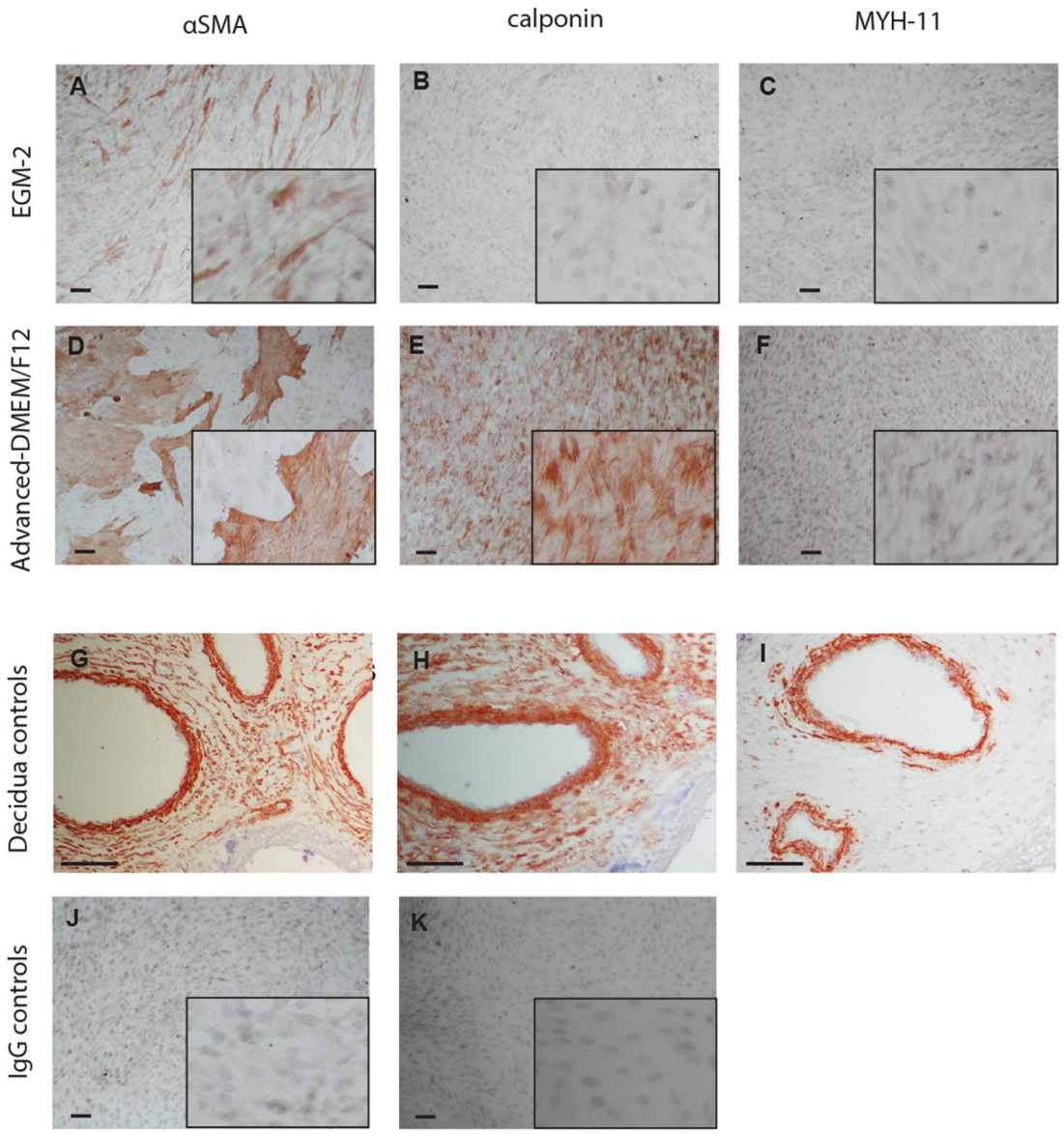

Figure 7. Human endothelial cells upregulate α-smooth muscle actin and calponin in culture. Photomicrographs of cultured endothelial cells after 14 days of culture in EGM-2 (**A**–**C**), or following transition to smooth muscle differentiation medium (advanced-DMEM/F12+TGF-β1) at Day 7 of culture (**D**–**F**). Positive control staining was undertaken in first trimester decidua (**G**–**I**). Irrelevant mouse (**J**) or rabbit (**K**) IgG antibodies were used as negative controls. Scale bars = 100 μm. SMA: smooth muscle actin; MYH-11: myosin heavy chain-11; EGM-2: endothelial growth media.

3.45%), which may reflect or be the cause of the reduced vascular density reported in FGR placentae (Gong *et al.*, 2011; Junaid *et al.*, 2014). FGR placentae also had an increased ratio of perivascular: endothelial cells, indicative of thicker vessel walls in these placentae. This finding is in line with the smaller vessel lumen to wall ratio previously reported in FGR placentae (Mitra *et al.*, 2000; Lu *et al.*, 2017), and functionally could be expected to negatively influence vessel permeability and exchange, decrease vascular compliance, and increase resistance to flow, as is seen in umbilical Doppler waveforms in FGR placentae (Papageorghiou *et al.*, 2004; Jones *et al.*, 2015). The placental microvasculature normally has far less pericyte coverage than the arterioles or venules in order to facilitate exchange (Harris *et al.*, 2020), and thus increased proportions of pericytes associated with these microvessels in FGR could also have important impacts on placental nutrient and oxygen exchange function. Finally, pericytes play a role in stabilizing nascent blood vessels, and endothelial cell sprouting involves the detachment of pericytes to enable endothelial cell reorganization when new vessels are formed (Adams and Alitalo, 2007). Therefore, if present from earlier in gestation, an increased perivascular cell: endothelial cell ratio could contribute to the reduction in branching angiogenesis in FGR placentae.

In this work, we confirmed the existence of a population of $CD31^{low}$ endothelial cells in term placentae, but, interestingly, found these cells were rare in the first trimester. It is important to acknowledge that both arterial and venous systems, that would be functionally mature by term, exist within the placental villi and could potentially explain the presence of two subpopulations of endothelial cells observed in this work. Low CD31 expression has previously been used to identify a 'Meso-Endothelial Bipotent Progenitor' in normal term placentae. This cell was hypothesized to represent an ancestral cell capable of differentiating into either endothelial or mesenchymal lineages (Shafiee *et al.*, 2018). However, whilst both endothelial and mesenchymal cells were initially observed in cultures, after passaging only mesenchymal cells were present (Shafiee *et al.*, 2018). An

Table 5. Summary of human villus core cell changes between first trimester, term and foetal growth restriction placentae.

Endothelial cells (CD31+CD34+)	First trimester (3.92%) < Term (10.83%)	Term (10.83%) = FGR (3.45%)
Perivascular cells (CD146+CD271+)	First trimester (10.3%) < Term (67.24%)	Term (67.24%) = FGR (56.93%)
Myofibroblast-like cells (CD26+CD90+)	First trimester (31.28%) < Term (24.83%)	Term (24.83%) = FGR (29.64%)
Stromal cells (Podoplanin+CD36+CD142+)	First trimester (47.75%) >Term (4.37%)	Term (4.37%) = FGR (12.01%)
Mesenchymal (CD73+CD90+)	First trimester (14.02%) >Term (N/A)	

FGR: foetal growth restriction; N/A: not applicable.

alternative interpretation of this data is that CD31^{low} endothelial cells may be undergoing, or predisposed to undergo, EndMT, a reversible process involving endothelial loss of cell–cell junctional/adhesin properties and an increase in invasive and migratory characteristics (Pinto *et al.*, 2016). Here, our 23-colour panel was able to characterize CD31^{low} endothelial cells in term placentae more fully than previous reports, demonstrating that they co-expressed the mesenchymal markers CD26 and CD90. In particular, the high expression of CD26 on CD31^{low} endothelial cells supports their predisposition to undergo EndMT, as knock out or inhibition of CD26 has previously been reported to silence TGF-β2 induced signalling, and subsequently inhibit EndMT (Shi *et al.*, 2015).

The reduced endothelial cell abundance and increased perivascular: endothelial ratio in FGR raises the possibility that a higher proportion of cells are undergoing EndMT in this disorder (Swietlik, 2016). As our work in term placentae suggested that CD31^{low} endothelial cells may be predisposed to EndMT, we then sought to quantify and characterize this population in early gestation placentae for the first time. However, in contrast to term placentae, CD31^{low} cells were very rare in the first trimester. Thus, we next asked—'despite very few CD31^{low} cells, are first trimester endothelial cells still susceptible to EndMT'? Indeed, first trimester endothelial cells spontaneously underwent EndMT *in vitro*, changing morphology, downregulating endothelial markers, and upregulating mesenchymal markers. This aligns with earlier evidence suggesting that first trimester CD31^{+} endothelial cells upregulated αSMA in culture (Aplin *et al.*, 2015; Swietlik, 2016). Interestingly, we only saw αSMA upregulation after culture in smooth muscle medium, suggesting that different culture conditions may induce different mesenchymal phenotypes. Whilst EndMT and vascular regression could be considered counterintuitive to early expansion of the vascular network, this process could facilitate branching angiogenesis and vascular rearrangement/morphogenesis by downregulating endothelial junctional markers and promoting a migratory phenotype (Welch-Reardon *et al.*, 2014). Finally, whilst CD31^{low} cells were rare in the first trimester, there was a large number of CD144$^{low/-}$ endothelial cells and, as loss of this marker is also observed during EndMT (Pinto *et al.*, 2016), this population could be fulfilling a similar role to that of CD31^{low} cells at term. However, CD144$^{low/-}$ cells did not express the mesenchymal markers seen on CD31^{low} cells (CD90, CD26). Further work is needed to elucidate the different pathways involved in initiating EndMT across gestation.

In summary, this work characterized the phenotype of human placental vascular and stromal populations in depth, demonstrating differences between first trimester and term that depict how the placenta matures into a highly functional vascular organ (Table 5). We demonstrated that placental EndMT gives rise to mesenchymal cells that can subsequently upregulate contractile markers, suggesting that this process could influence vascular compliance and feto-placental blood flow. The increase in the perivascular: endothelial cell ratio in FGR placentae, and depletion of endothelial cells, raises the possibility that EndMT is dysregulated in this disorder, and the capacity of first trimester endothelial cells to undergo EndMT highlights the potential role of this process from early in gestation, when the pathophysiology of FGR arises. As aberrant EndMT is a feature of some cardiovascular disorders (Sánchez-Duffhues *et al.*, 2018; Kovacic *et al.*, 2019) and can be targeted therapeutically, these findings warrant further investigation of the role of EndMT in normal placental vascular development and in the pathophysiology of FGR.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Data availability

Spectrally unmixed flow cytometry data (FSC files) are available in FLOWRepository (http://flowrepository.org/id/FR-FCM-Z6UK and http://flowrepository.org/id/FR-FCM-Z4TJ).

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Authors' roles

A.L.B.—data curation, analysis, drafting original manuscript, conceptualization. L.W.C.—interpretation of data, review and editing. A.E.S.B.—conceptualization, interpretation of data, resources, supervision, review. J.L.J.—review and editing, conceptualization, interpretation of data, funding, resources, supervision.

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Conflict of interest

The authors declare no conflicts of interest.

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